Receptor signaling: **When dimerization is not enough**

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Activation of receptors that signal via tyrosine kinase domains has been thought to involve receptor dimerization and transphosphorylation of juxtaposed catalytic domains. Recent results suggest things might be more complex – specific intersubunit conformational changes within a dimer can also be important.

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For a wide range of transmembrane receptors, the crucial event that initiates a signaling pathway inside the cell is tyrosine phosphorylation. One large class of receptors — the receptor tyrosine kinases — have an intrinsic tyrosine kinase domain. Many other receptors, including the large family of cytokine receptors, lack intrinsic catalytic activity but associate inside the cell with non-receptor tyrosine kinases, which act as receptor catalytic subunits. The accepted model for activation of both types of receptor is that ligand-induced receptor dimerization juxtaposes the cytoplasmic tyrosine kinase domains, resulting in transphosphorylation of regulatory tyrosine residues in the activation loop of the partner molecule and thereby promoting phosphorylation of target proteins and initiating signaling cascades. Intrasubunit phosphorylation of the key regulatory tyrosines is presumably precluded by steric factors, making oligomerization a requirement for activation [1]. Recent studies on the erythropoietin (Epo) receptor (EpoR) and the ErbB2/Neu receptor tyrosine kinase, however, suggest that while dimerization is required, it is not always sufficient for receptor activation [2–7].

EpoR, a member of cytokine receptor superfamily, associates constitutively with the non-receptor tyrosine kinase JAK2 via its cytoplasmic domain. Dogma has it that unliganded EpoR exists as inactive monomers in association with JAK2, and that ligand activates EpoR by inducing dimerization and thereby juxtaposing the JAK2 molecules. Neu/ErbB2, an orphan member of the epidermal growth factor (EGF) receptor family, has intrinsic tyrosine kinase activity. Neu*, an oncogenic form of Neu with a point mutation in the transmembrane domain — replacing valine 664 by glutamate — undergoes constitutive dimerization and can induce cell transformation [8]. Glutamate 664 in Neu* is protonated, thus allowing an interchain hydrogen bond to form between two mutant transmembrane domains [9]. These observations suggested that dimerization is sufficient for Neu activation.

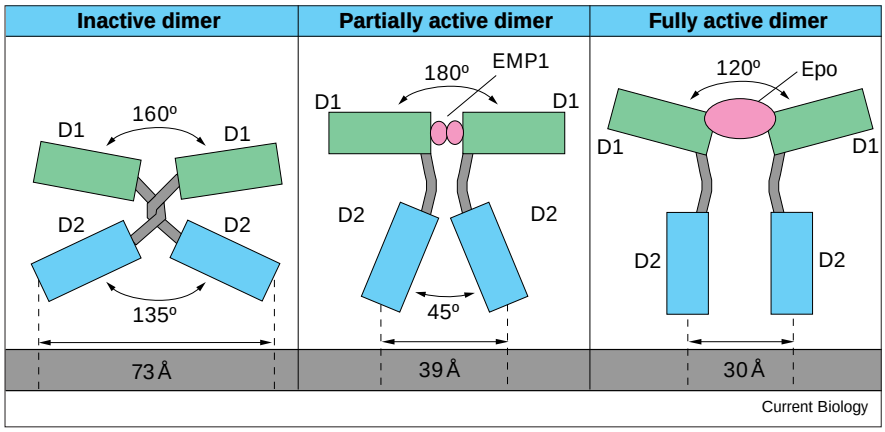
The EpoR consists of an extracellular domain, referred to as the Epo-binding protein (EBP), a single transmembrane domain and a cytoplasmic domain. The EBP has two fibronectin type III domains, D1 and D2, connected via an interdomain region (Figure 1). Despite extensive evidence for the ligand-induced dimerization model, Livnah *et al.* [4] surprisingly found that unliganded EBP crystallized as a dimer. Interestingly, the unliganded EBP dimer was seen to adopt a very different conformation than that of EBP complexed with the peptide agonist Epo-mimetic peptide 1 (EMP1) [3,4] (Figure 1). In the unliganded dimer, the two D2 domains are oriented away from each other at an angle of 135°, and the two transmembrane domains are separated by 73 Å. In contrast, in the EMP1-bound dimer, the D2 domains are oriented away from each other at an angle of 45° and the transmembrane domains are separated by only 39 Å. Moreover, results from another group had shown that, in the Epo–$(EBP)_2$ complex, the two D2 domains are aligned parallel and the transmembrane domains are separated by a mere 30 Å [10] (Figure 1).

Taken together, these results led Livnah *et al.* [4] to conclude that the cytoplasmic domains of the unliganded dimers will be kept sufficiently far apart to prevent interaction of the two associated JAK2 molecules, thereby reducing background signaling. Epo or EMP1 binding would result in receptor reorientation, bringing the transmembrane and cytoplasmic domains closer together, thus facilitating transphosphorylation of the JAK2 molecules. The results of complementation assays with protein fragments confirmed that unliganded EpoR molecules do indeed exist as dimers in which the cytoplasmic domains are kept apart, and that EpoR-associated JAK2 molecules can physically interact with each other only when EpoR binds to EMP1 or Epo [5]. These results indicate that at least a fraction of unliganded EpoR molecules exist as inactive dimers *in vivo*. The implication is that dimerization by itself is insufficient for EpoR activation, and that activation of this receptor involves domain reorientation within a preformed dimer complex.

There is evidence for conformation-dependent activation of the EpoR dimer in the published literature. For instance, the antagonist peptide EMP33 inactivates EpoR but still induces EpoR dimerization. Interestingly,

Figure 1

Relative orientations of the extracellular – 'Epo-binding protein' (EBP) – region of the Epo receptor when dimerized in unliganded (left), EMP1-liganded (middle) or Epo-liganded (right) states [3,4,10]. The plane of the membrane is orthogonal to the paper. Dashed lines project from the carboxyl termini of the D2 extracellular domains through the membrane. The liganded complex contains two EMP1 molecules. Note that the two D2 domains are aligned and separated differently in the three complexes.



the relative orientation of the two EBP molecules complexed with EMP33 is different than that of the two EBP molecules complexed with EMP1 or Epo [2,3,10]. Furthermore, only a small fraction of the bivalent monoclonal antibodies that bind EBP are activating [11]. It appears, therefore, that EpoR molecules can form dimers in which the two subunits have different relative orientations associated with 'on', 'off' or various 'intermediate' activity states. A further indication that the EpoR transmembrane domain might be designed to dimerize comes from the observation that substitution of residue arginine 129, which lies close to the EpoR transmembrane domain, by cysteine led to dimerization via an intermolecular disulfide bond, and that the resulting dimer was constitutively active [12]. The spacing between the transmembrane domains in this dimer is presumably less than 30 Å. It will be important to obtain structural information on the full-length liganded and unliganded EpoR dimers to determine the true transmembrane and cytoplasmic domain spacing in the native protein.

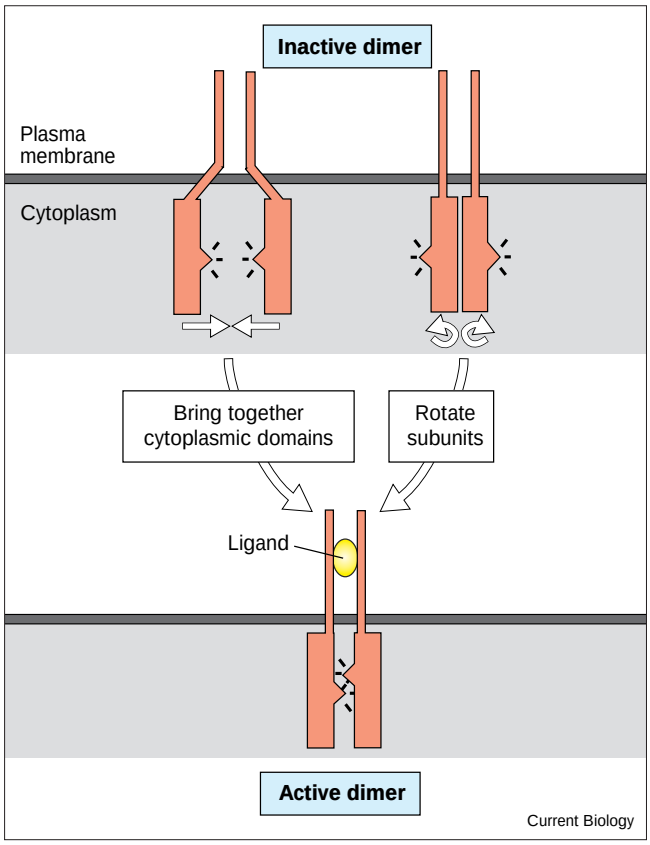
Will what we have learned from these observations about EpoR turn out to be generally applicable to cytokine receptor activation? The extracellular domains of cytokine receptors are highly conserved [13], but no structure has yet been reported of the unliganded extracellular domain of a cytokine receptor other than EpoR. One intriguing indication that the findings with EpoR might be generally applicable has come from the observation that c-Mpl, a cytokine receptor for thrombopoietin, was constitutively activated by the introduction of cysteines into its membrane-proximal extracellular domain, but not by the introduction of cysteines nearer its amino terminus [14].

The findings on EpoR activation may have broader implications for the activation of receptor tyrosine kinases in general. The transmembrane domains of most growth factor receptor tyrosine kinases appear to have a conserved dimerization motif [15], so, in principle at least, they also could exist as inactive dimers in the absence of ligand. Furthermore, in the case of the ErbB2/Neu receptor tyrosine kinase, it is likely that dimerization is required, but not necessarily sufficient, for activation. Burke *et al.* [6] showed previously that a chimeric form of the Neu receptor containing the transmembrane domain of glycophorin A dimerizes, but does not transform cells and has only slightly increased kinase activity. The same group [7] has more recently shown that, when cysteine residues were introduced at various membrane-proximal positions of Neu's extracellular domain, only a subset of the resulting receptor dimers had transforming activity. Furthermore, the cysteines in the activating mutants all mapped to one face of a predicted α helix, the same face as valine 664 which is replaced by glutamate in the oncogenic Neu.

These findings indicate that productive dimerization of Neu is orientation-dependent — that is, a precise rotational coupling of the monomers is essential to achieve activation [7]. The possible existence of inactive receptor tyrosine kinase dimers is underscored by the finding that the insulin and insulin-like growth factor 1 (IGF-1) receptor tyrosine kinases exist as constitutively disulfide-bonded, inactive $(\alpha\beta)_2$ dimers in the unliganded state. Binding of ligand to these covalent dimers stimulates their tyrosine kinase activity [16]. In the case of the insulin and IGF-1 receptors, therefore, ligand binding must induce a conformational change in preformed dimers that activates their catalytic domains.

The notion that simple dimerization is insufficient for receptor activation is also applicable to other types of receptor of both eukaryotic and prokaryotic origins. One example is the tumor necrosis factor (TNF) receptor. TNF binding is thought to result in dimerization or trimerization of free, monomeric receptor molecules, leading to oligomerization of the cytoplasmic domain and initiation of

Figure 2



A model for the activation of inactive receptor dimers by rotational coupling. Top left: an inactive receptor tyrosine kinase dimer, in which the two catalytic domains are kept apart. Top right: an inactive receptor tyrosine kinase dimer in which the catalytic domains are in close proximity but in a wrong or suboptimal orientation (facing away from each other). Bottom: an active, liganded receptor dimer in which the two catalytic domains are brought into close proximity in a productive conformation that allows transphosphorylation and activation.

signaling. Interestingly, however, the extracellular domain of unliganded TNF receptor-1 was found to exist as a dimer in two distinct crystal forms [17,18]. In one crystal form, the two extracellular domains were seen to form an antiparallel dimer, with the TNF binding site occluded; the cytoplasmic domains would be predicted to be separated in complete dimers by more than 100 Å, preventing signaling. In the second form, which can in principle bind TNF, the extracellular domain forms a parallel dimer, bringing the transmembrane domains close together. TNF receptor-1 oligomerization has recently been shown to be actively blocked by binding of the newly identified protein dubbed 'silencer of death domains' (SODD) to its cytoplasmic domain [19], and this could be a general mechanism for regulating receptor oligomerization.

Moving to prokaryotes, the bacterial transmembrane chemoreceptors, such as the aspartate receptor Tar, provide a further example. Unliganded Tar is found as a non-covalent dimer. Binding to its chemoattractant ligand does not change the oligomeric state of Tar, but it does alter the activity of the associated CheA histidine kinase. This leads, via a typical 'two-component' signal transduction pathway, to counterclockwise flagellar movement and smooth swimming. The fact that Tar receptors signal without a monomer–dimer transition once again shows that dimerization per se can be insufficient for receptor activation, and that intersubunit conformational changes and subunit reorientation can additionally be involved [20].

What are the advantages of the unliganded receptor being pre-assembled into dimers and there being a strict requirement for subunit reorientation to activate the receptor? The presence of unliganded dimers may facilitate subsequent formation of ligand–receptor complexes by increasing the effective receptor concentration. This may be particularly relevant when a ligand binds to the first receptor subunit with a high affinity, but to the second subunit with a much lower affinity, as in the case of the Epo–EpoR interaction [21]. The insufficiency of simple dimerization for receptor activation may serve as a fail-safe mechanism to prevent accidental activation when two receptor monomers collide during diffusion on the cell surface.

There are at least two possible ways that relative domain orientation can restrict transphosphorylation in a receptor dimer (Figure 2). Non-activating dimerization may keep the kinase domains physically apart, as suggested by the structure of the unliganded EpoR dimer [4,5]. Alternatively, non-activating dimerization may fix the kinase domains in close proximity but in a wrong or suboptimal orientation — that is, facing away from each other. This possibility is suggested by the presence of a non-activating dimerization interface in Neu [7] and the less-than-optimal signaling efficiency of different EMPs [2,3,10]. In both cases, the inactive receptor dimer can be activated by a rotational coupling process that brings the tyrosine kinase catalytic domains closer together and/or turns them around, so that each subunit can present itself as a suitable substrate for the catalytic domain of the partner molecule. This, in principle, is similar to the 'piston-swing' model proposed for the regulation of Tar activity [20].

The finding that relative subunit orientation is critically important for receptor dimer activation presents new challenges, as well as opportunities, for the design of small molecule agonists and antagonists. It may prove difficult to discover small molecules that induce, not only receptor dimerization, but also the correct conformational reorientation. Using random phage display peptide libraries and affinity selection methods, small peptidyl agonists, which have no sequence homology to the natural ligand Epo but are capable of inducing dimerization and signaling of

EpoR, have been successfully discovered. In general, however, these agonists are much less efficient in signaling than the natural ligand Epo [22,23]. As discussed above, these Epo-mimetic peptides do not orient the receptor dimers in the same way as Epo [3,10]. A peptide agonist that is as potent as the natural cytokine has, however, been found for the thrombopoietin receptor [24].

Very recently, SB 247464, a small dimeric, non-peptidyl mimic of granulocyte-colony stimulating factor, has been identified by functional screening [25]. SB 247464 is much less potent than the natural ligand, perhaps because the orientation of the two receptor subunits in the dimer is not optimal for activation. Nevertheless, the fact that suboptimal relative subunit orientations can generate dimers with graded activities may also serve as the basis for the discovery of small compounds that can fine-tune the activity of target molecules.

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